

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 1 025 849 A1

(12)

EUROPEAN PATENT APPLICATION

(43) Date of publication:

09.08.2000 Bulletin 2000/32

(21) Application number: **99204249.9**

(22) Date of filing: **14.12.1993**

(51) Int. Cl.⁷: **A61K 35/12, C12Q 1/00,**

C12Q 1/02, C12N 5/10,

C12N 15/00, C12N 15/52,

C07K 7/06

(84) Designated Contracting States:

**AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL
PT SE**

(30) Priority: **22.12.1992 US 994928**

28.04.1993 US 54714

23.06.1993 US 81673

(62) Document number(s) of the earlier application(s) in
accordance with Art. 76 EPC:

94905393.8 / 0 687 180

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Remarks:

- This application was filed on 10 - 12 - 1999 as a
divisional application to the application mentioned
under INID code 62.
- The biological material has been deposited with
CNCM Paris under number(s) I1275 & I1276

(54) **Methods for detection and treatment of individuals having abnormal cells expressing HLA-A2/tyrosinase peptide antigens**

(57) The invention relates to the identification of complexes of human leukocyte antigen molecules and tyrosinase derived peptides on the surfaces of abnormal cells. The therapeutic and diagnostic ramifications of this observation are the subject of the invention

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Description**FIELD OF THE INVENTION**

5 [0001] This invention relates to various therapeutic methodologies derived from the recognition that certain abnormal cells present complexes of human leukocyte antigens and peptides derived from tyrosinase on their surfaces. In addition, it relates to the ability to identify those individuals diagnosed with conditions characterized by cellular abnormalities whose abnormal cells present this complex, the presented peptides, and the ramifications thereof.

BACKGROUND AND PRIOR ART

[0002] The process by which the mammalian immune system recognizes and reacts to foreign or alien materials is a complex one. An important facet of the system is the T cell response. This response requires that T cells recognize and interact with complexes of cell surface molecules, referred to as human leukocyte antigens ("HLA"), or major histocompatibility complexes ("MHCs"), and peptides. The peptides are derived from larger molecules which are processed by the cells which also present the HLA/MHC molecule. See in this regard Male et al., *Advanced Immunology* (J.P. Lipincott Company, 1987), especially chapters 6-10. The interaction of T cell and complexes of HLA/peptide is restricted, requiring a T cell specific for a particular combination of an HLA molecule and a peptide. If a specific T cell is not present, there is no T cell response even if its partner complex is present. Similarly, there is no response if the specific complex is absent, but the T cell is present. This mechanism is involved in the immune system's response to foreign materials, in autoimmune pathologies, and in responses to cellular abnormalities. Recently, much work has focused on the mechanisms by which proteins are processed into the HLA binding peptides. See, in this regard, Barinaga, *Science* 257: 880 (1992); Fremont et al., *Science* 257: 919 (1992); Matsumura et al., *Science* 257: 927 (1992); Latron et al., *Science* 257: 964 (1992).

25 [0003] The mechanism by which T cells recognize cellular abnormalities has also been implicated in cancer. For example, in PCT application PCT/US92/04354, filed May 22, 1992, published on November 26, 1992, and incorporated by reference, a family of genes is disclosed, which are processed into peptides which, in turn, are expressed on cell surfaces, which can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., *Immunogenetics* 35: 145 (1992); van der Bruggen et al., *Science* 254: 1643 (1991), for further information on this family of genes.

[0004] In U.S. patent application Serial Number 938,334, the disclosure of which is incorporated by reference, non-peptides are taught which bind to the HLA-A1 molecule. The reference teaches that given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind one HLA molecule, but not others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

40 [0005] The enzyme tyrosinase catalyzes the reaction converting tyrosine to dehydroxyphenylalanine or "DOPA" and appears to be expressed selectively in melanocytes (Muller et al., *EMBOJ* 7: 2715 (1988)). An early report of cDNA for the human enzyme is found in Kwon, U.S. Patent No. 4,898,814. A later report by Bouchard et al., *J. Exp. Med.* 169: 2029 (1989) presents a slightly different sequence. A great deal of effort has gone into identifying inhibitors for this enzyme, as it has been implicated in pigmentation diseases. Some examples of this literature include Jinbow, WO9116302; Mishima et al., U.S. Patent No. 5,077,059, and Nazzaropor, U.S. Patent No. 4,818,768. The artisan will be familiar with other references which teach similar materials.

45 [0006] None of these references teach or suggest, however, that tyrosinase may be treated in a manner similar to a foreign antigen or a TRAP molecule - i.e., it has now been found that in certain cellular abnormalities, such as melanoma, tyrosinase is processed and a peptide derived therefrom forms a complex with HLA molecules on certain abnormal cells. These complexes are recognized by cytolytic T cells ("CTLs"), which then lyse the presenting cells. The ramifications of this surprising and unexpected phenomenon are the subject of the invention, which is described in greater detail in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

55 [0007]

Figure 1 describes, collectively, cell lysis studies. In particular:

Figure 1A shows lysis of cell line LB24;
 Figure 1B shows lysis of cell line SK29-MEL;
 Figure 1C shows lysis of cell line LB4.MEL;
 Figure 1D shows lysis of cell line SK23.MEL;
 5 Figure 1E shows lysis of cell line LE516.MEL;
 Figure 1F shows lysis studies on NK target K562;
 Figure 1G shows lysis of autologous, EBV-B transformed cells;
 Figure 1H shows lysis of the loss variant in Figure 1F after transfection with a gene for HLA-A2;
 Figure 1I shows lysis of autologous IEBV- β transformed cells.
 10 Figure 2 presents studies of TNF release of CTL IVSB.
 Figure 3 depicts studies of TNF release of CTL 210/9.
 Figure 4 depicts the recognition of the peptide YMNGTMSQV by cytolytic T cell clone CTL-IVSB but not cytolytic T cell clone CTL 2/9.
 Figure 5 shows that the peptide YMNGTMSQV is not recognized by cytolytic T cell clone CTL 210/9.
 15 Figure 6 shows the results obtained when TNF release assays were carried out on various cells, including those which present HLA-B44 on their surface.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Example 1

[0008] Melanoma cell lines SK 29-MEL (also referred to in the literature as SK MEL-29) and LB24-MEL, which have been available to researchers for many years, were used in the following experiments.

[0009] Samples containing mononuclear blood cells were taken from patients AV and LB24-MEL (these patients 25 were also the source of SK 29-MEL and LB24-MEL, respectively). The melanoma cell lines were contacted to the mononuclear blood cell containing samples. The mixtures were observed for lysis of the melanoma cell lines, this lysis indicating that cytolytic T cells ("CTLs") specific for a complex of peptide and HLA molecule presented by the melanoma cells were present in the sample.

[0010] The lysis assay employed was a chromium release assay following Herin et al., Int. J. Cancer 39:390-396 30 (1987), the disclosure of which is incorporated by reference. The assay, however, is described herein. The target melanoma cells were grown *in vitro*, and then resuspended at 10^7 cells/ml in DMEM, supplemented with 10 mM HEPES and 30% FCS, and incubated for 45 minutes at 37°C with 200 μ Ci/ml of Na(51 Cr)O₄. Labelled cells were washed three times with DMEM, supplemented with 10 mM Hepes. These were then resuspended in DMEM supplemented with 10 mM Hepes and 10% FCS, after which 100 μ l aliquots containing 10^3 cells, were distributed into 96 well microplates. 35 Samples of PBLs were added in 100 μ l of the same medium, and assays were carried out in duplicate. Plates were centrifuged for 4 minutes at 100g, and incubated for four hours at 37°C in a 5.5% of CO₂ atmosphere.

[0011] Plates were centrifuged again, and 100 μ l aliquots of supernatant were collected and counted. Percentage of 51 Cr release was calculated as follows:

$$\% \text{ } ^{51}\text{Cr release} = \frac{(ER-SR)}{(MR-SR)} \times 100$$

where ER is observed, experimental 51 Cr release, SR is spontaneous release measured by incubating 10^3 labeled cells 45 in 200 μ l of medium alone, and MR is maximum release, obtained by adding 100 μ l 0.3% Triton X-100 to target cells.

[0012] Those mononuclear blood samples which showed high CTL activity were expanded and cloned via limiting dilution, and were screened again, using the same methodology.

[0013] The same method was used to test target K562 cells. When EBV-B cells were used, the only change was the replacement of DMEM medium by Hank's medium, supplemented with 5% FCS.

50 [0014] These experiments led to isolation of CTL clone "IVSB" from patient AV and CTL clone 210/9 from patient LB24.

[0015] Figure 1 presents the results of these assays, in panels A, B, G and I. Specifically, it will be seen that both CTLs lysed both melanoma cell lines, and that there was no lysis of the K562 and EBV-B cell lines.

Example 2

55 [0016] The CTLs described were tested against other melanoma cell lines to determine whether their target was shared by other melanoma cell lines. Lysis as described in Example 1 was studied for lines LB4.MEL, SK23.MEL (also

known as SK MEL-23), and LE516.MEL. Figure 1, panels C, D and E shows that the clones did lyse these lines.

[0017] The tested lines are known to be of type HLA-A2, and the results suggested that the CTLs are specific for a complex of peptide and HLA-A2. This suggestion was verified by testing a variant of SK 29-MEL which has lost HLA-A2 expression. Figure 1, panel F shows these results. Neither clone lysed the HLA-loss variant. When the variant was transfected with the HLA-A2 gene of SK29-MEL, however, and retested, lysis was observed. Thus, it can be concluded that the presenting molecule is HLA-A2.

Example 3

[0018] Once the presenting HLA molecule was identified, studies were carried out to identify the molecule, referred to hereafter as the "tumor rejection antigen precursor" or "TRAP" molecule which was the source of the presented peptide.

[0019] To do this, total RNA was isolated from cell line SK29-MEL.1, which is a subclone of SK29-MEL. The RNA was isolated using an oligo-dT binding kit, following well recognized techniques. Once the total RNA was secured, it was transcribed into cDNA, again using standard methodologies. The cDNA was then ligated to EcoRI adaptors and cloned into the EcoRI site of plasmid pcDNA-I/Amp, in accordance with manufacturer's instructions. The recombinant plasmids were then electroporated into JM101 *E. coli* (electroporation conditions: 1 pulse at 25 μ farads, 2500 V).

[0020] The transfected bacteria were selected with ampicillin (50 μ g/ml), and then divided into 700 pools of 200 clones each. Each pool represented about 100 different cDNAs, as analysis showed that about 50% of plasmids contained an insert. Each pool was amplified to saturation, and plasmid DNA was isolated via alkaline lysis, potassium acetate precipitation and phenol extraction, following Maniatis et al., in Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, N.Y., 1982). Cesium gradient centrifugation was not used.

Example 4

[0021] The amplified plasmids were then transfected into eukaryotic cells. Samples of COS-7 cells were seeded, at 15,000 cells/well into tissue culture flat bottom microwells, in Dulbecco's modified Eagles Medium ("DMEM") supplemented with 10% fetal calf serum. The cells were incubated overnight at 37°C, medium was removed and then replaced by 30 μ l/well of DMEM medium containing 10% Nu serum, 400 μ g/ml DEAE-dextran, 100 μ M chloroquine, 100 ng of plasmid pcDNA-I/Amp-A2 and 100 ng of DNA of a pool of the cDNA library described supra. Plasmid pcDNA-I/Amp-A2 contains the HLA-A2 gene from SK29-MEL. Following four hours of incubation at 37°C, the medium was removed, and replaced by 50 μ l of PBS containing 10% DMSO. This medium was removed after two minutes and replaced by 200 μ l of DMEM supplemented with 10% of FCS.

[0022] Following this change in medium, COS cells were incubated for 48 hours at 37°C. Medium was then discarded, and 2000 cells of either of the described CTL clones were added, in 100 μ l of Iscove medium containing 10% pooled human serum. When done 210/9 was used, the medium was supplemented with 25 U/ml of IL-2. Supernatant was removed after 24 hours, and TNF content was determined in an assay on WEHI cells, as described by Traversari et al., Immunogenetics 35: 145-152 (1992), the disclosure of which is incorporated by reference.

[0023] Of 700 wells tested with IVSB, 696 showed between 0.6 and 4 pg of TNF per ml. The remaining four wells contained between 10 and 20 pg/ml of TNF. Homologous wells tested with CTL 210/9 showed similar, clearly higher values. Figures 2 and 3 present these data.

Example 5

[0024] Three of the four pools identified as high producers (numbers "123", "181" and "384") were selected for further experiments. Specifically, the bacteria were cloned, and 570 bacteria were tested from each pool. Plasmid DNA was extracted therefrom, transfected into a new sample of COS cells in the same manner as described supra, and the cells were again tested for stimulation of CTL 210/9 and CTL IVSB. A positive clone was found in pool 123 ("p123.B2"), and one was found in pool 384 ("p384.C6"). Convincing evidence that the transfected cells were recognized by CTLs was obtained by carrying out a comparative test of COS cells transfected with cDNA and the HLA-A2 gene, and COS cells transfected only with HLA-A2. TNF release in CTL supernatant was measured by testing it on WEHI cells. The optical density of the surviving WEHI cells was measured using MTT. Results are presented in Table 1:

Table 1

	cDNA (123.B2) + HLA-A2 DNA	no cDNA+ HLA-A2
Run 1	0.087	0.502
Run 2	0.108	0.562

[0025] The values for WEHI OD's correspond to 24 pg/ml of TNF for cDNA and HLA-A2, versus 2.3 pg/ml for the control.

[0026] The plasmids from the positive clones were removed, and sequenced following art known techniques. A sequence search revealed that the plasmid insert was nearly identical to the cDNA for human tyrosinase, as described by Bouchard et al., J. Exp. Med. 169: 2029 (1989), the disclosure of which is incorporated by reference. Thus, a normally occurring molecule (i.e., tyrosinase), may act as a tumor rejection antigen precursor and be processed to form a peptide tumor rejection antigen which is presented on the surface of a cell, in combination with HLA-A2, thereby stimulating lysis by CTL clones. The nucleic sequence of the identified molecule is presented as SEQ ID NO: 1.

Example 6

[0027] Prior work reported by Chomez et al., Immunogenetics 35: 241 (1992) has shown that small gene fragments which contain a sequence coding for an antigenic peptide resulted in expression of that peptide. This work, which is incorporated by reference in its entirety, suggested the cloning of small portions of the human tyrosinase cDNA described supra and in SEQ ID NO: 1. Using the methodologies described in examples 1-5, various fragments of the cDNA were cotransfected with a gene for HLA-A2 in COS-7 cells, and TNF release assays were performed. These experiments led to identification of an approximately 400 base pair fragment which, when used in cotransfection experiments, provoked TNF release from cytolytic T cell clone CTL IVSB discussed supra, shown to be specific for HLA-A2 presenting cells. The 400 base fragment used corresponded to bases 711 to 1152 of SEQ ID NO: 1. The amino acid sequence for which the fragment codes was deduced, and this sequence was then compared to the information provided by Hunt et al., Science 255: 1261 (1992), and Falk et al., Nature 351: 290 (1991), the disclosures of which are both incorporated by reference in their entirety. These references discuss consensus sequences for HLA-A2 presented peptides. Specifically, Hunt discusses nonapeptides, where either Leu or Ile is always found at the second position, Leu being the "dominant residue". The ninth residue is described as always being a residue with an aliphatic hydrocarbon side chain. Val is the dominant residue at this position. Hunt, discusses a strong signal for Leu and an intermediate signal for Met at the second position, one of Val, Leu, Ile or Thr at position 6, and Val or Leu at position 9, with Val being particularly strong. On the basis of the comparison, nonapeptides were synthesized and then tested to see if they could sensitize HLA-A2 presenting cells. To do so, tyrosinase loss variant cell lines SK29-MEL 1.218 and T202LB were used. Varying concentrations of the tested peptides were added to the cell lines, together with either of cytolytic T cell clone CTL IVSB or cytolytic T cell clone CTL 2/9. Prior work, described supra, had established that the former clone lysed tyrosinase expressing cells which present HLA-A2, and that the latter did not.

[0028] The tyrosinase loss variants were incubated for one hour in a solution containing ^{51}Cr , at 37°C, either with or without anti HLA-A2 antibody MA2.1, which was used to stabilize empty HLA-A2 molecules. In the tests, cells were washed four times, and then incubated with varying dilutions of the peptides, from 100 μM down to 0.01 μM . After 30 minutes, effector cells were added at an E/T ratio of 40/1 and four hours later, 100% of supernatant were collected and radioactivity counted.

[0029] Figure 4 shows the results obtained with nonapeptide Tyr Met Asn Gly Thr Met Ser Gln Val. (SEQ ID NO: 2).

[0030] This peptide, referred to hereafter as SEQ ID NO: 2, corresponds to residues 1129-1155 of the cDNA sequence for tyrosinase presented in SEQ ID NO: 1. Complexes of HLA-A2 and this peptide are recognized by CTL clone CTL IVSB.

[0031] In a parallel experiment, it was shown that CTL clone CTL 210/9, derived from patient LB24, did not recognize the complexes of HLA-A2 and the peptide of SEQ ID NO: 2, although it did recognize complexes of HLA-A2 and a tyrosinase derived peptide. Thus, tyrosinase is processed to at least one additional peptide which, when presented by HLA-A2 molecules, is recognized by CTL clones.

Example 7

[0032] In a follow-up experiment, a second gene fragment which did not encode the peptide of SEQ ID NO: 2 was used. This fragment began at base 1 and ended at base 1101 of SEQ ID NO: 1 (i.e. the EcoRI-SphI fragment). Cytolytic T cell clone CTL 210/9, discussed *supra*, was tested against COS-7 cells transfected with this fragment in the manner described *supra*. CTL IVSB was also tested. These results, showed that LB24-CTL 210/9 recognized an antigen on the surface of HLA-A2 cells transfected with this fragment, but CTL IVSB did not. Thus, a second tumor rejection antigen peptide is derived from tyrosinase.

Example 8

[0033] In order to further define the tumor rejection antigen recognized by LB24-CTL 210/9, the following experiments were carried out.

[0034] A second fragment, corresponding to bases 451-1158 of SEQ ID NO: 1 was transfected into COS cells together with a gene for HLA-A2, and TNF release assays were carried out. This sequence provoked TNF release from clone SK29-CTL IVSB (20 pg/ml), but not from LB24-CTL 210/9 (3.8 pg/ml). These results confirmed that the two CTL clones recognize different peptides, and that the peptide recognized by LB24-CTL 210/9 must be encoded by region 1-451.

Example 9

[0035] The tyrosinase derived peptide coded for by cDNA fragment 1-451 was analyzed for consensus sequences known to bind HLA-A2. The peptides corresponding to these consensus sequences were synthesized, and tested for their ability to sensitize HLA-A2 presenting cells. To do so, two tyrosinase negative melanoma cell lines were used (i.e., NA8-MEL, and MZ2-MEL 2.2 transfected with HLA-A2), and cell line T2, as described by Salter et al, Immunogenetics 21: 235-246 (1985), were used.

[0036] The cells were incubated with ^{51}Cr , and monoclonal antibody MA.2.1, which is specific to HLA-A2 for 50 minutes at 37°C, followed by washing (see Bodmer et al., Nature 342: 443-446 (1989), the disclosure of which is incorporated by reference in its entirety). Target cells were incubated with various concentrations of the peptides, and with either of LB 24-CTL clones 210/5 or 210/9. The percent of chromium release was measured after four hours of incubation.

[0037] The peptide Met Leu Leu Ala Val Leu Tyr Cys Leu Leu (SEQ ID NO: 3) was found to be active.

[0038] In further experiments summarized here, CTL-IVSB previously shown to recognize YMNGTMSQV, did not recognize the peptide of SEQ ID NO: 3.

[0039] The results are summarized in Tables 2-4 which follow:

Table 2

	PEPTIDE	
	YMNGTMSQV (1120-1155)	MLLAVLYCLL (25-54)
SK29-CTL-IVSB	+	-
LB24-CTL-210/5	-	+
LB24-CTL-210/9	-	+

Table 3

3J93-Lysis of MZ2-2.2-A2 sensitized with tyrosinase peptides by LB24-CTL 210/5 and 210/9, and SK29-CTL IVSB			
Effectors	Peptides	Dose	MZ2.2.2.A2 +anti-A2 *
LB24-CTL. 210/5 (44:1)	MLLAVLYCLL (LAUS 17-5)	10μM	18
		3	17
		1	16
	YMNGTMSQV (MAINZ)	30M	1
		10	1
		3	1
LB24-CTL. 210/9 (30:1)	MLLAVLYCLL (LAUS 17-5)	10μM	18
		3	17
		1	15
	YMNGTMSQV (MAINZ)	30M	1
		10	1
		3	1
SK29CTL. IVSB (40:1)	MLLAVLYCLL (LAUS 17-5)	10μM	1
		3	1
		1	1
	YMNGTMSQV (MAINZ)	30μM	68
		10	68
		3	62
CTL cells were added at the indicated (E:T) ratio. The % specific Cr51 release was mesured after 4h incubation			

* Target cells were incubated with Cr51 and mono-Ab MA2.1 (anti-HLA-A2) for 50 min, then washed 3 times.

They were incubated with various concentrations of peptides for 30 min

Table 4

8J93 : Tout of tyrosinase peptides recognized by LB24-CTL 210/5 and 210/9 or SK29-CTL IVSB (% Cr51 specific release)

Effectors	Peptides	Dose	NA8-MEL *	MZ2-2.2: A2	T2
LB24-CTL. 210/5 (41:1)	MLLAVLYCLL (LAUS 17-5)	10 μ M	30	31	36
		3	23	27	35
		1	17	20	26
		300nM	6	17	16
		100	2	8	5
		30	3	5	2
		0	0	0	0
		10 μ M	14	19	21
		3	13	17	20
		1	9	14	13
LB24-CTL. 210/9 (26:1)	MLLAVLYCLL (LAUS 17-5)	300nM	3	9	5
		100	1	1	1
		30	0	1	0
		0	0	1	0
		10 μ M	46	46	59
		3	38	44	52
		1	27	40	46
		300nM	14	22	34
		100	3	13	21
		30	1	9	10
SK29-CTL. IVSB (42:1)	YMNGTMSQV (MAINZ)	10	1	3	3
		3	0	3	4
		1	0	1	0
		0	0	4	0
		spt. rel.	339	259	198
		max-spt	2694	1693	1206
		%	11	13	14
		0	0	4	0
		10 μ M	46	46	59
		3	38	44	52

CTL cells were added at the indicated E:T ratio.

The % specific Cr51 release was mesured after 4h incubation

* Target cells were incubated with Cr51 end mono-Ab MA2.1 (anti-HLA-A2) for 50', then washed 3 times.

They were incubated with various concentrations of peptides for 30'

Example 10

[0040] Additional experiments were carried out using CTL clone 22/31. This clone had previously been shown to lyse subline MZ2-MEL.43 from autologous melanoma cell line MZ2-MEL, but did not lyse other sublines, such as MZ2-MEL 3.0 and MZ2-MEL 61.2, nor did it lyse autologous EBV transformed B cells, or killer cell line K562 (see Van den Eynde et al., Int. J. Cancer 44: 634-640 (1989)). The antigen presented by MZ2-MEL.43 is referred to as antigen C.

[0041] In prior work including that reported in the parent of this application, it was found that the tyrosinase gene encodes an antigen recognized by autologous CTLs on most HLA-A2 melanoma. Expression of this gene on sublines of cell line MZ2-MEL was tested by PCR amplification. Clone MZ2-MEL.43 was found to be positive, whereas other MZ2-MEL clones, such as MZ2-MEL.3.0 were negative. Correlation of expression of the tyrosinase gene, and antigen MZ2-C, suggested that MZ2-C might be a tumor rejection antigen derived from tyrosinase, and presented by an HLA molecule expressed by MZ2-MEL. This cell line does not express HLA-A2, which would indicate that if a tyrosinase derived peptide were presented as a TRA, a second HLA molecule was implicated.

[0042] Studies were carried out to identify which HLA molecule presented antigen C to CTL 22/31. To determine this, cDNA clones of the HLA molecules known to be on the cell surface, i.e., HLA-A29, HLA-B37, HLA-B 44.02, and HLA-C clone 10, were isolated from an MZ2-MEL.43 cDNA library, and then cloned into expression vector pcDNA1/Amp. Recipient COS 7 cells were then transfected with one of these constructs or a construct containing HLA-A1, plus cDNA coding for tyrosinase (SEQ ID NO: 1). The cotransfection followed the method set forth above. One day later CTL 22/31 was added, and 24 hours later, TNF release was measured by testing cytotoxicity on WEHI-164-13, following Traversari et al, *supra*. Figure 6 shows that TNF was released by CTL 22/31 only in the presence of cells transfected with both HLA-B44 and tyrosinase. The conclusion to be drawn from this is that HLA-B44 presents a tyrosinase derived tumor rejection antigen.

[0043] The foregoing experiments demonstrate that tyrosinase is processed as a tumor rejection antigen precursor, leading to formation of complexes of the resulting tumor rejection antigens with a molecule on at least some abnormal cells, for example, melanoma cells with HLA-A2 or HLA-B44 phenotype. The complex can be recognized by CTLs, and the presenting cell lysed. This observation has therapeutic and diagnostic ramifications which are features of the invention. With respect to therapies, the observation that CTLs which are specific for abnormal cells presenting the aforementioned complexes are produced, suggests various therapeutic approaches. One such approach is the administration of CTLs specific to the complex to a subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CTLs *in vitro*. Specifically, a sample of cells, such as blood cells, are contacted to a cell presenting the complex and capable of provoking a specific CTL to proliferate. The target cell can be a transfectant, such as a COS cell of the type described *supra*. These transfectants present the desired complex on their surface and, when combined with a CTL of interest, stimulate its proliferation. So as to enable the artisan to produce these CTLs, vectors containing the genes of interest, i.e., pcDNA-1/Amp1 (HLA-A2), and p123.B2 (human tyrosinase), have been deposited in accordance with the Budapest Treaty at the Institut Pasteur, under Accession Numbers I1275 and I1276, respectively. COS cells, such as those used herein are widely available, as are other suitable host cells.

[0044] To detail the therapeutic methodology, referred to as adoptive transfer (Greenberg, J. Immunol. 136(5): 1917 (1986); Reddel et al., Science 257: 238 (7-10-92); Lynch et al., Eur. J. Immunol. 21: 1403-1410 (1991); Kast et al., Cell 59: 603-614 (11-17-89)), cells presenting the desired complex are combined with CTLs leading to proliferation of the CTLs specific thereto. The proliferated CTLs are then administered to a subject with a cellular abnormality which is characterized by certain of the abnormal cells presenting the particular complex. The CTLs then lyse the abnormal cells, thereby achieving the desired therapeutic goal.

[0045] The foregoing therapy assumes that at least some of the subject's abnormal cells present one or more of the HLA/tyrosinase derived peptide complexes. This can be determined very easily. For example CTLs are identified using the transfectants discussed *supra*, and once isolated, can be used with a sample of a subject's abnormal cells to determine lysis *in vitro*. If lysis is observed, then the use of specific CTLs in such a therapy may alleviate the condition associated with the abnormal cells. A less involved methodology examines the abnormal cells for HLA phenotyping, using standard assays, and determines expression of tyrosinase via amplification using, e.g., PCR. The fact that a plurality of different HLA molecules present TRAs derived from tyrosinase increases the number of individuals who are suitable subjects for the therapies discussed herein.

[0046] Adoptive transfer is not the only form of therapy that is available in accordance with the invention. CTLs can also be provoked *in vivo*, using a number of approaches. One approach, i.e., the use of non-proliferative cells expressing the complex, has been elaborated upon *supra*. The cells used in this approach may be those that normally express the complex, such as irradiated melanoma cells or cells transfected with one or both of the genes necessary for presentation of the complex. Chen et al., Proc. Natl. Acad. Sci. USA 88: 110-114 (January, 1991) exemplifies this approach, showing the use of transfected cells expressing HPV E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. In these systems, the gene of interest is carried by, e.g., a *Vaccinia* virus or the bacteria BCG, and the materials *de facto* "infect" host cells. The cells which result present the complex of interest, and are recognized by autologous CTLs, which then proliferate. A similar effect can be achieved by combining tyrosinase itself with an adjuvant to facilitate incorporation into HLA-A2 presenting cells. The enzyme is then processed to yield the peptide partner of the HLA molecule.

[0047] The foregoing discussion refers to "abnormal cells" and "cellular abnormalities". These terms are employed in their broadest interpretation, and refer to any situation where the cells in question exhibit at least one property which

indicates that they differ from normal cells of their specific type. Examples of abnormal properties include morphological and biochemical changes, e.g. Cellular abnormalities include tumors, such as melanoma, autoimmune disorders, and so forth.

5 [0048] The invention also provides a method for identifying precursors to CTL targets. These precursors are referred to as tumor rejection antigens when the target cells are tumors, but it must be pointed out that when the cell characterized by abnormality is not a tumor, it would be somewhat misleading to refer to the molecule as a tumor rejection antigen. Essentially, the method involves identifying a cell which is the target of a cytolytic T cell of the type discussed supra. Once such a cell is identified, total RNA is converted to a cDNA library, which is then transfected into a cell sample capable of presenting an antigen which forms a complex with a relevant HLA molecule. The transfectants are contacted with the CTL discussed supra, and again, targeting by the CTL is observed (lysis and/or TNF production).
10 These transfectants which are lysed are then treated to have the cDNA removed and sequenced, and in this manner a precursor for an abnormal condition, such as a tumor rejection antigen precursor, can be identified.

[0049] Other aspects of the invention will be clear to the skilled artisan and need not be repeated here.

15 [0050] The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

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EP 1 025 849 A1

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Claims

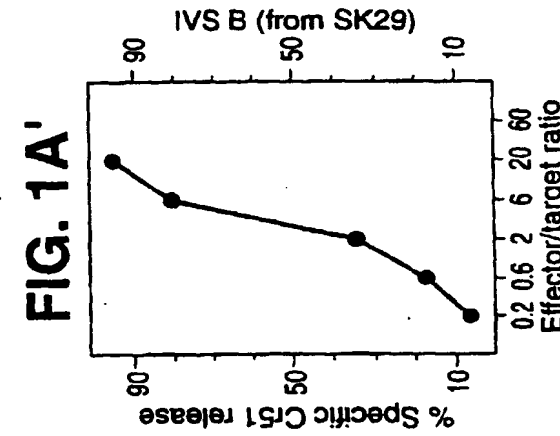
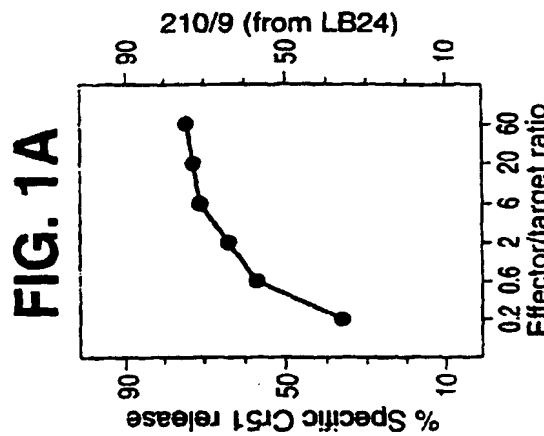
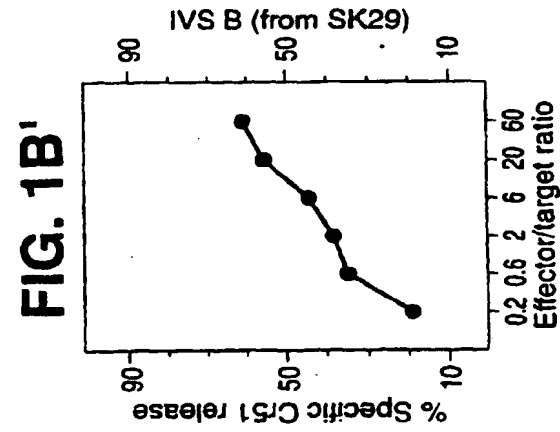
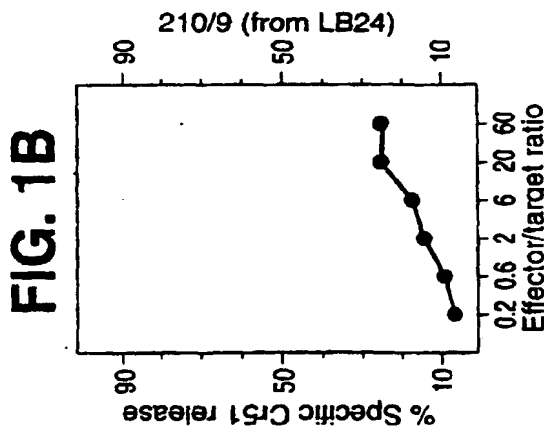
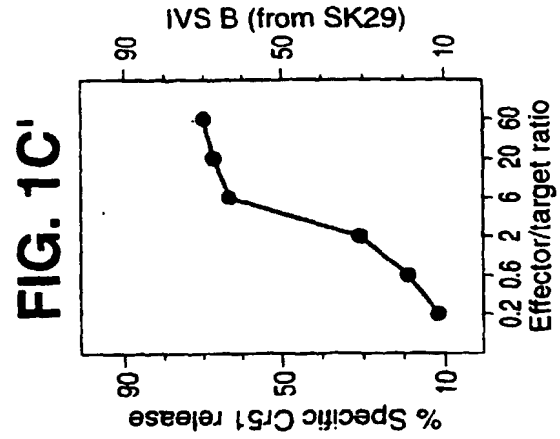
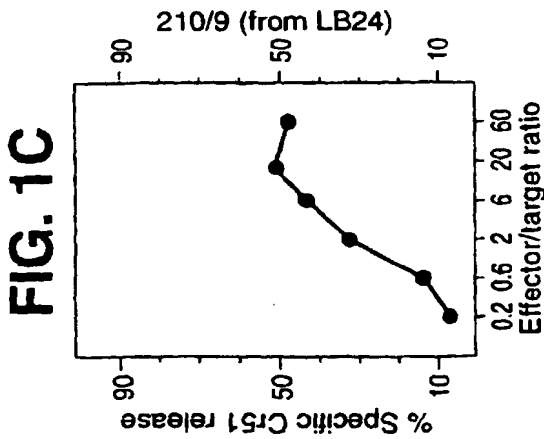
1. An isolated peptide consisting of the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val.
- 50 2. A method for determining presence of melanoma cell specific cytolytic T cells in a sample as a diagnosis of melanoma in a subject from whom said sample has been taken, comprising contacting a mononuclear blood cell containing sample from a subject of interest with melanoma cells which present, on their surface, complexes of HLA-A2 molecules and the peptide Tyr Met Asn Gly Thr Met Ser Gln Val, and determining one of (i) proliferation of cytolytic T cells in said mononuclear blood cell containing sample and (ii) lysis of said melanoma cells, as a deter-
 55 mination of presence of melanoma cell specific cytolytic T cells in said sample, said presence being indicative of melanoma in said subject.
3. A method according to claim 2, comprising determining proliferation of cytolytic T cells by measuring tumor necro-

sis factor release.

4. A method according to claim 2 or 3, comprising determining lysis of said cells by determining release of radio labelled chromium.
5. A method according to any one of claims 2-4, wherein said cells which present said complexes have been transfected with at least one of (i) a nucleic acid molecule which codes for an HLA molecule and (ii) a nucleic acid molecule which codes for tyrosinase.
6. A method according to any one of claims 2-4, wherein said cells have been transfected with both of (i) a nucleic acid molecule which codes for an HLA molecule and (ii) a nucleic acid molecule which codes for tyrosinase.
7. A composition for use in a therapeutic treatment, comprising an isolated peptide consisting of the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
8. A composition for use in a therapeutic treatment, comprising a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
9. A composition for use in a therapeutic treatment, comprising a vector able to provoke a cytolytic response to cells presenting a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu, said vector carrying (i) a nucleic acid molecule which codes for tyrosinase, or a portion thereof that includes the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu, and, optionally, (ii) a nucleic acid molecule which codes for the HLA molecule.
10. A composition as claimed in one any of claims 7 to 9, wherein said treatment is tumour therapy.
11. A composition as claimed in claim 10, wherein said treatment is of melanoma.
12. A composition comprising cells presenting a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu on their surface, wherein said cells have been transfected with one or both of (i) a nucleic acid molecule which codes for an HLA molecule and (ii) a nucleic acid molecule which codes for tyrosinase, or a portion thereof that includes the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
13. A composition as claimed in claim 8, comprising cells presenting said complex on their surface.
14. A composition as claimed in claim 13, wherein the cells are transfectants.
15. A composition as claimed in claim 14, wherein said cells have been transfected with one or both of (i) a nucleic acid molecule which codes for an HLA molecule and (ii) a nucleic acid molecule which codes for tyrosinase, or a portion thereof that includes the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
16. A composition as claimed in claim 12 or claim 15, wherein said cells have been transfected with one or both of the vectors pcDNA-1/Amp1 and p123.B2, deposited at the Institute Pasteur under accession numbers I1275 and I1276 respectively.
17. A composition as claimed in any one of claims 12 to 16, wherein the cells are non-proliferative.
18. A composition as claimed in any one of claims 12 to 17, wherein the cells are melanoma cells.
19. A composition as claimed in any one of claims 7 to 11 and 17, wherein said composition is for use in a therapeutic treatment involving administering the composition to a subject with a cellular abnormality characterised by certain of the abnormal cells presenting a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
20. A composition as claimed in any one of claims 12 to 18, wherein said composition is for use in a therapeutic treat-

ment involving contacting a sample of blood cells with the composition, to generate Cytolytic T cells specific for a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu, and administering the Cytolytic T cells to a subject with a cellular abnormality characterised by certain of the abnormal cells presenting the complex.

21. A composition as claimed in any of claims 8 to 20, wherein the HLA molecule is an HLA-A2 molecule.
22. Use of a composition as defined in any of claims 7 to 21 in the preparation of a medicament for use in tumor therapy.
23. Use of a composition as defined in any of claims 7 to 21 in the preparation of a medicament for use in the therapy of a cellular abnormality which is characterised by certain of the abnormal cells presenting a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
24. A use as claimed in claim 22, wherein the tumor is a melanoma.
25. A use as claimed in any one of claims 22 to 24, wherein the therapy involves administration of Cytolytic T cells specific to a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu to a subject.
26. A use as claimed in claim 25, wherein the Cytolytic T cells are generated *in vitro*.
27. A use as claimed in claim 26, wherein the composition is as defined in any one of claims 12 to 18 and the Cytolytic T cells are generated by contacting a sample of blood cells with the composition.
28. A use as claimed in any one of claims 22 to 24, wherein the therapy involves generating a CTL response *in vivo*.
29. A use as claimed in claim 28, wherein said treatment involves administering the composition to a subject with a cellular abnormality characterised by certain of the abnormal cells presenting a complex of an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
30. A use as claimed in any of claims 23 to 29, wherein the HLA molecule is an HLA-A2 molecule.
31. A method of generating Cytolytic T cells specific for a complex comprising an HLA molecule and a peptide having the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu, said method comprising the step of contacting a sample of blood cells to cells presenting said complex.
32. A method as claimed in claim 31, wherein the HLA molecule is HLA-A2.
33. A method as claimed in claim 31 or claim 32, wherein the cells have been transfected with at least one of (i) a nucleic acid molecule which codes for HLA-A2 and (ii) a nucleic acid molecule which codes for tyrosinase, or a portion thereof that includes the amino acid sequence Tyr Met Asn Gly Thr Met Ser Gln Val, or Met Leu Leu Ala Val Leu Tyr Cys Leu Leu.
34. A method of preparing a composition for use in a therapeutic treatment, comprising carrying out a method as claimed in any one of claims 31 to 33.
35. A method as claimed in claim 34, wherein said treatment is of cancer, preferably melanoma.
36. A composition as claimed in any one of claims 7-21, a use as claimed in any one of claims 22-30, or a method as claimed in any one of claims 31-35, wherein the amino acid sequence is Tyr Met Asn Gly Thr Met Ser Gln Val.



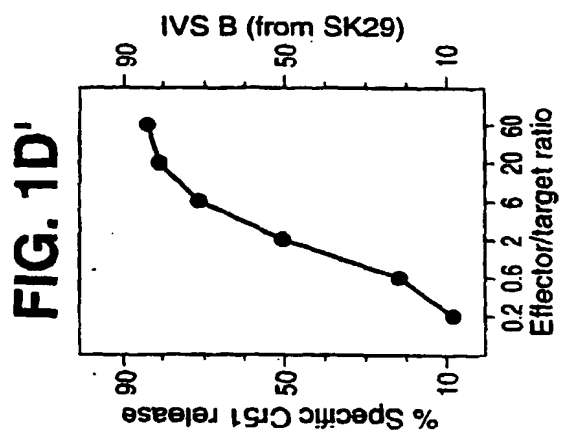
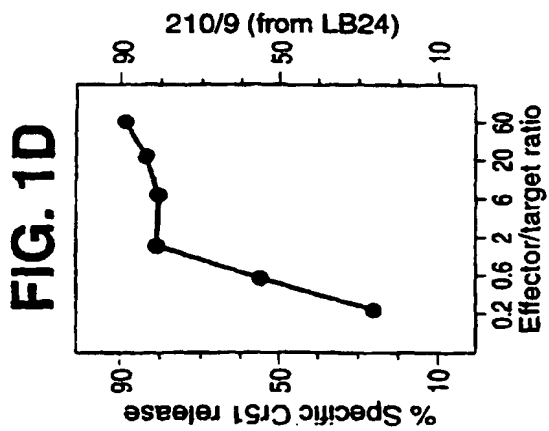
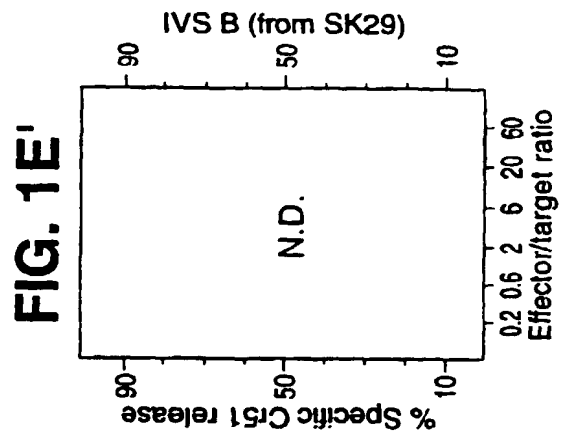
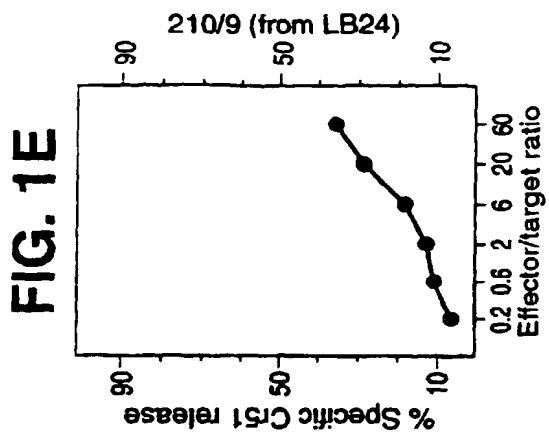


FIG. 1F

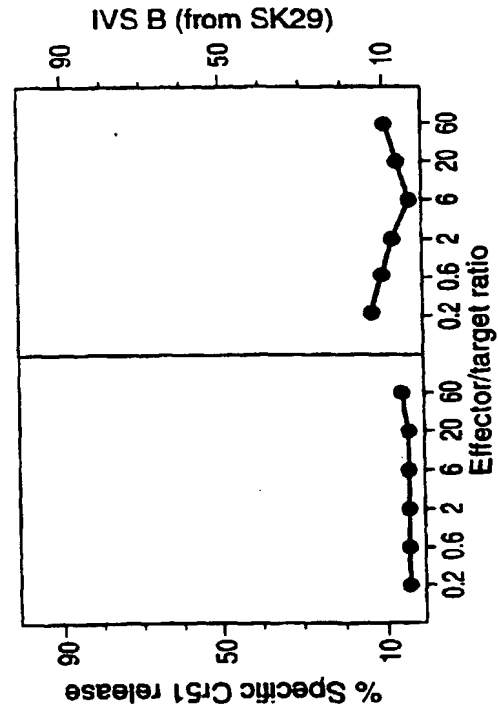
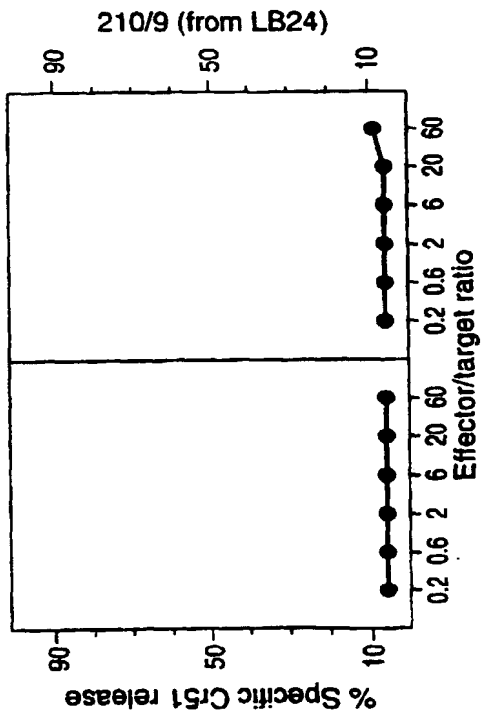
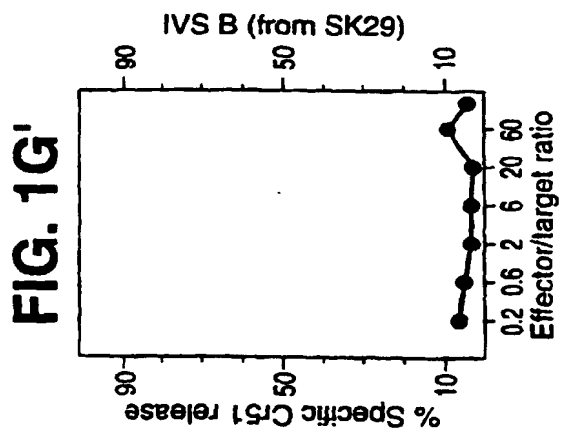
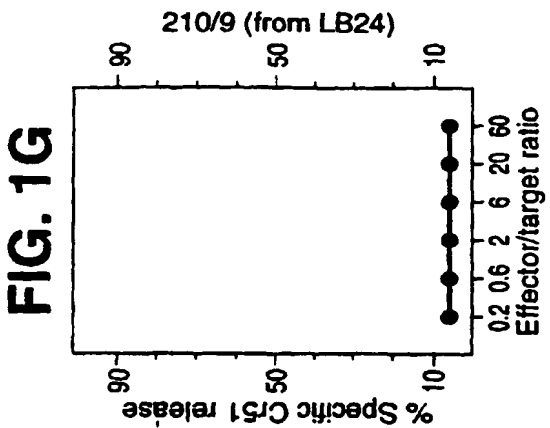
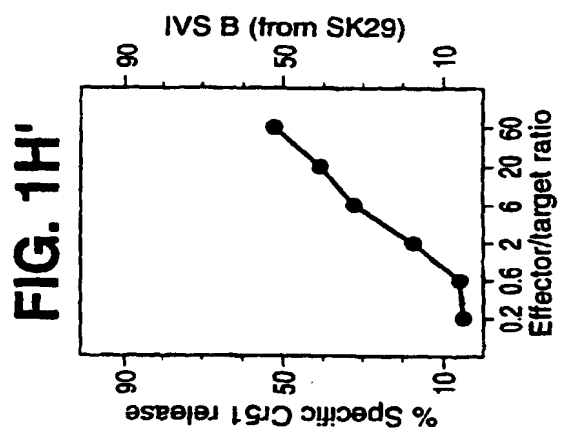
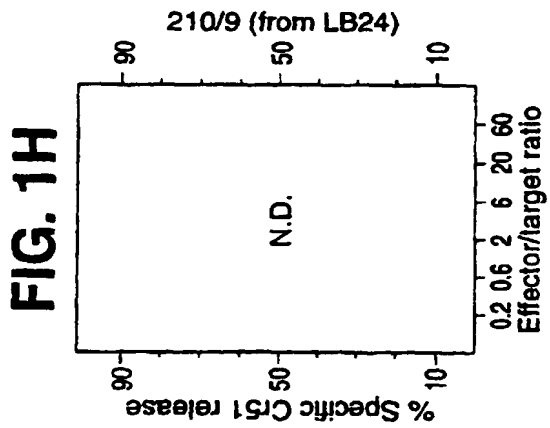
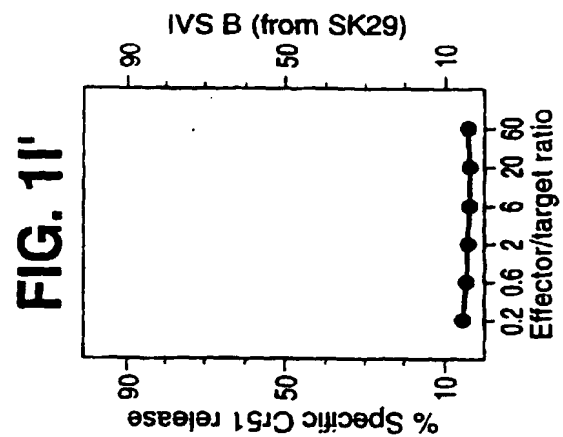
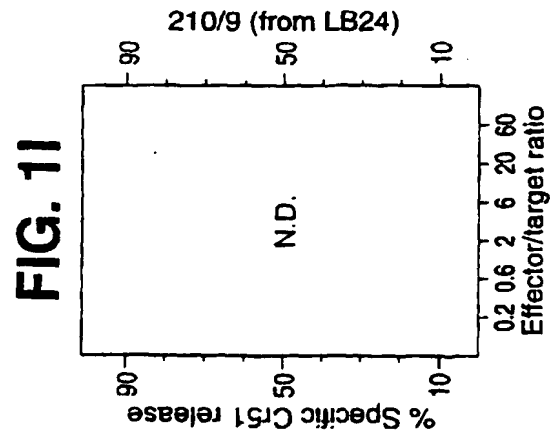
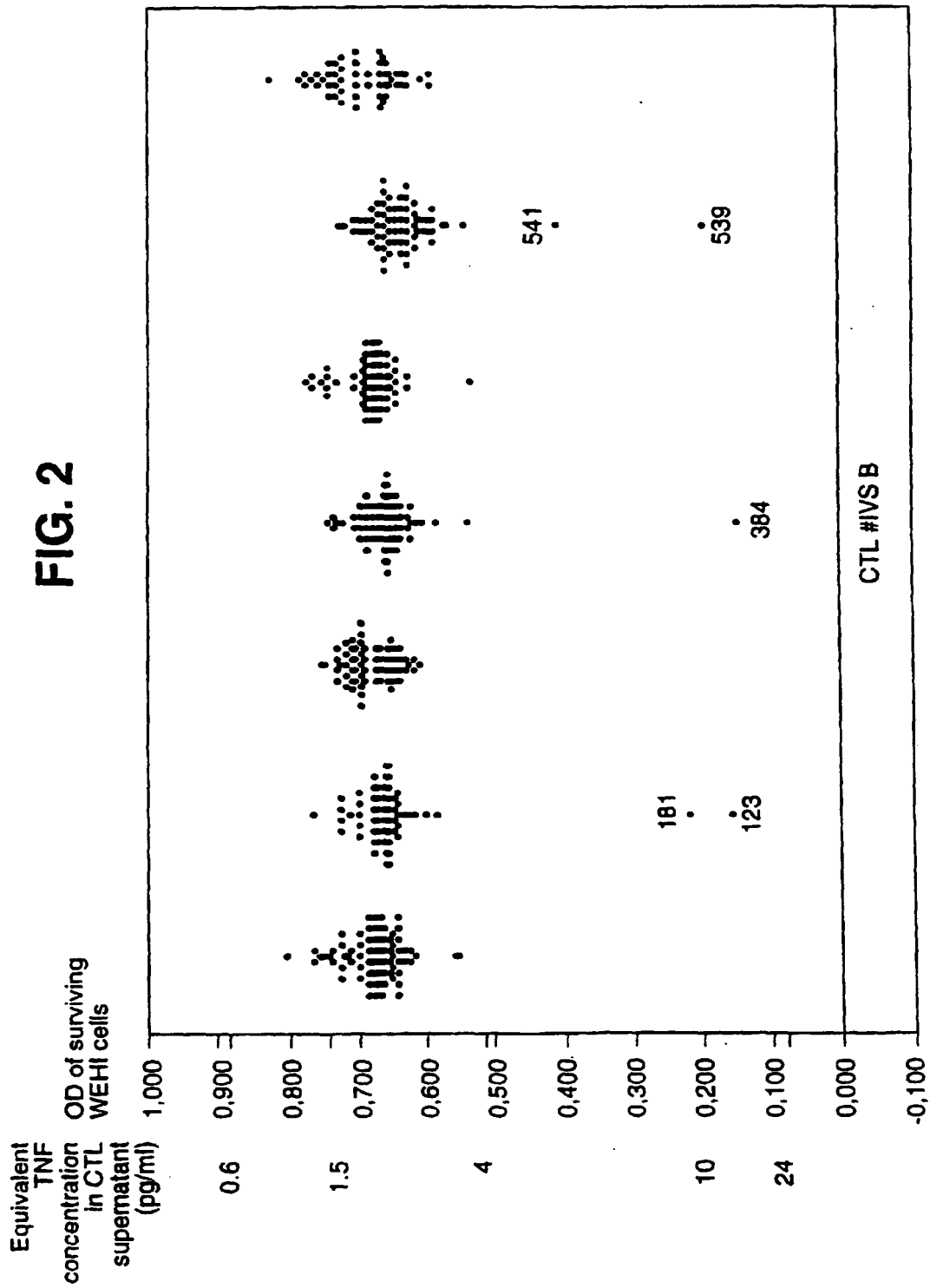


FIG. 1F'





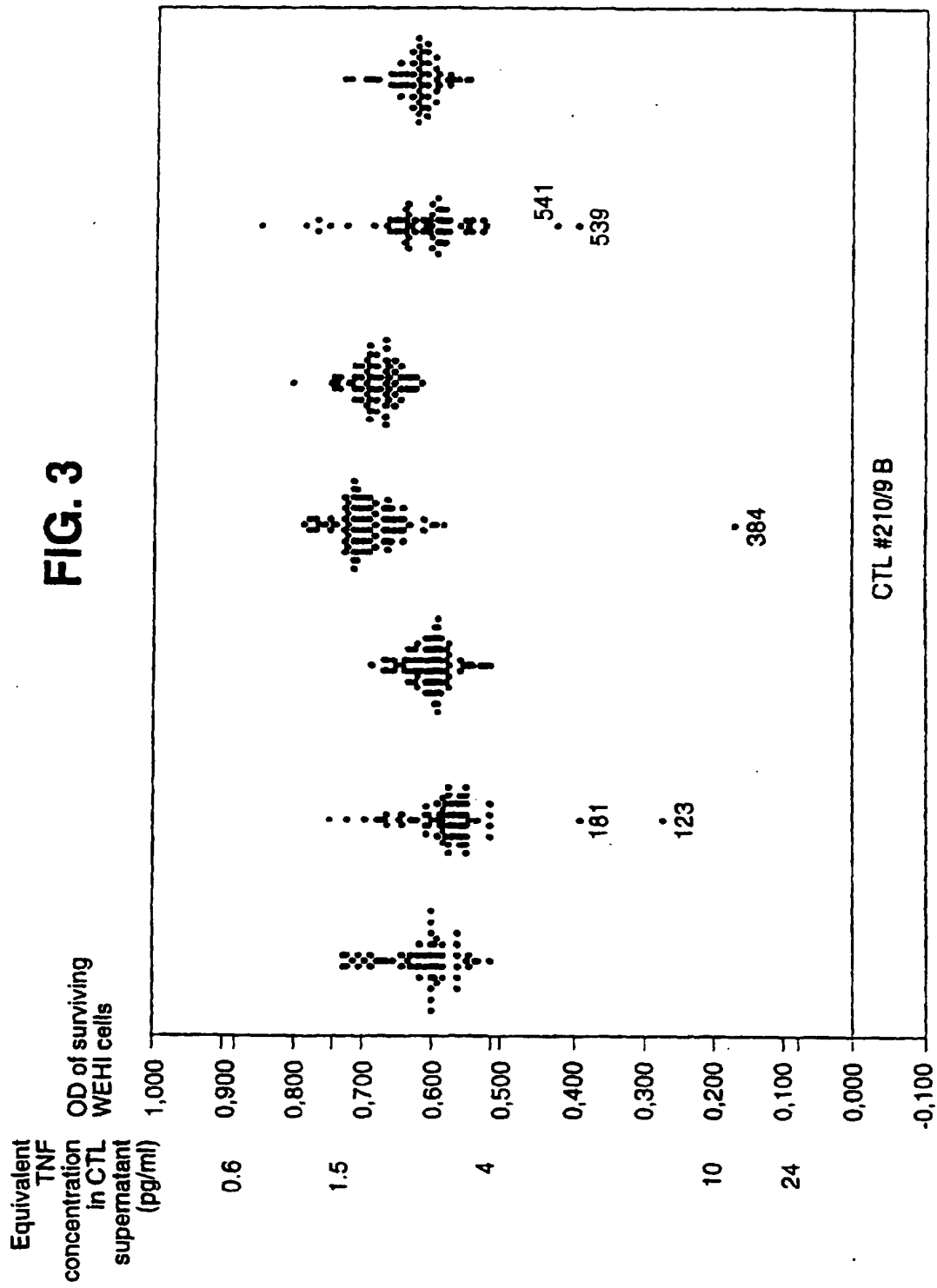


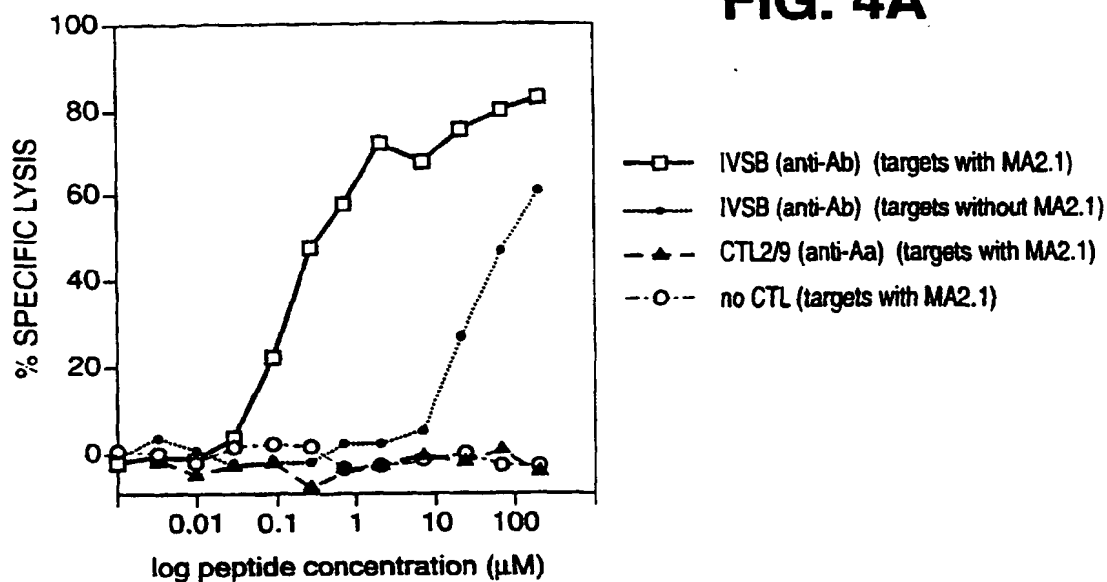
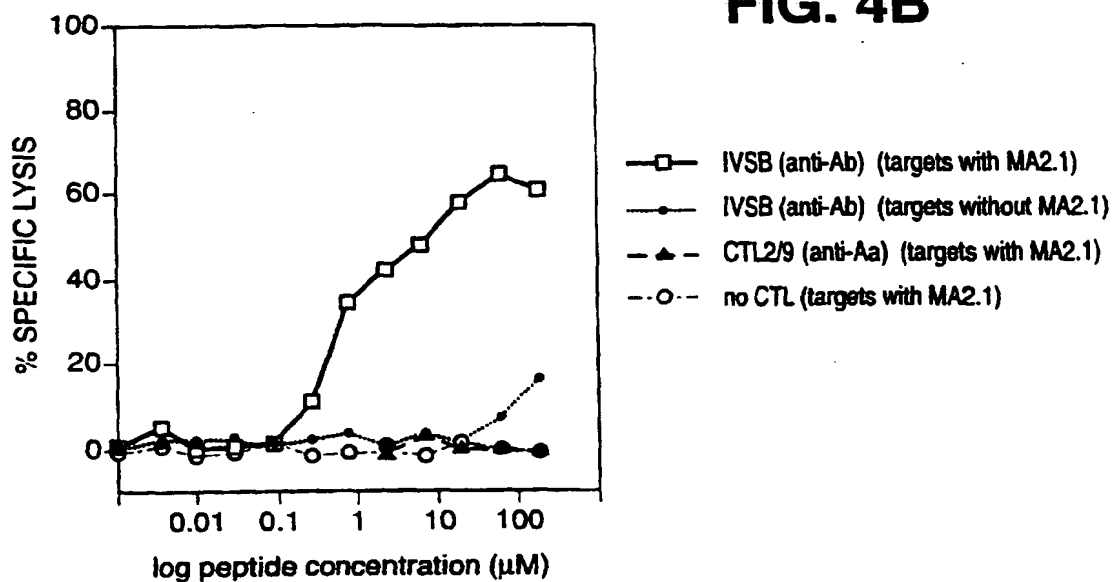
FIG. 4A**FIG. 4B**

FIG. 5

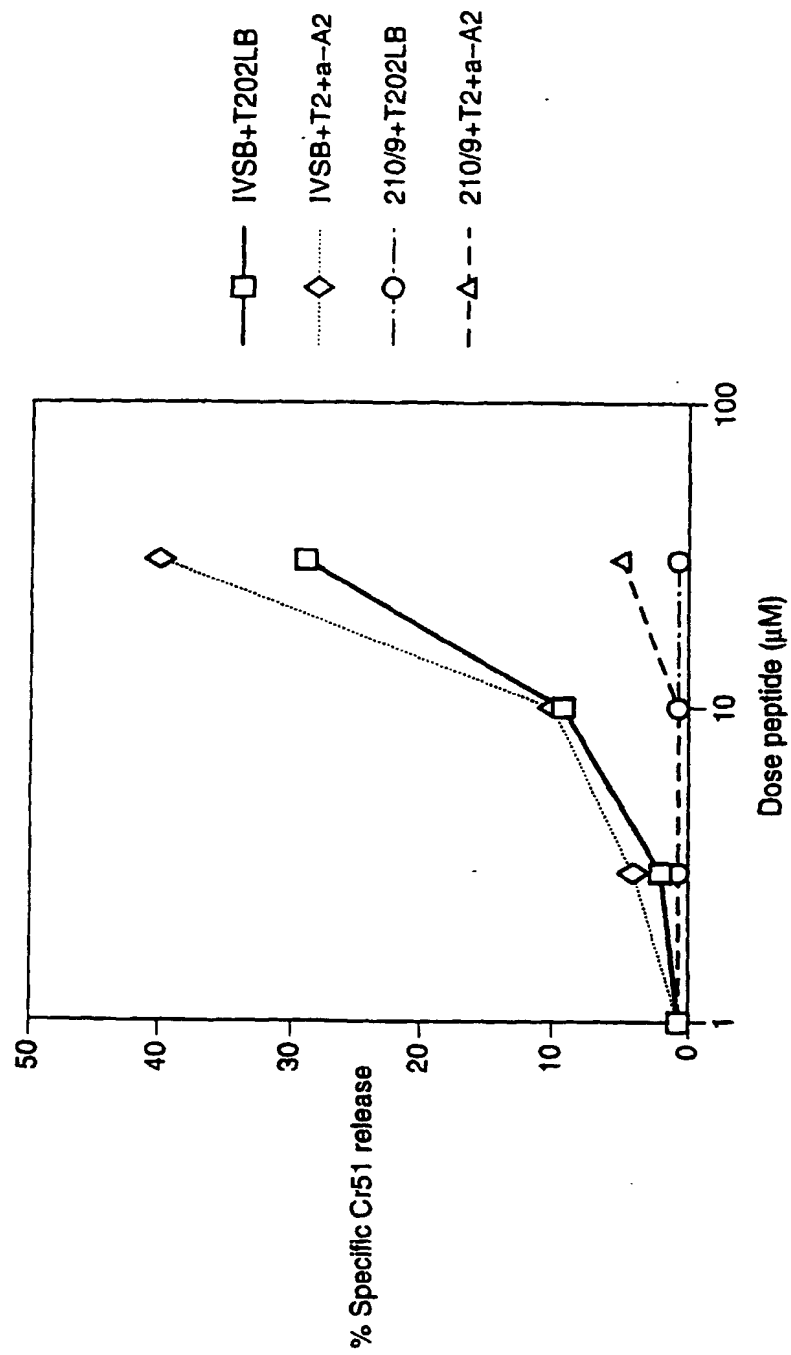
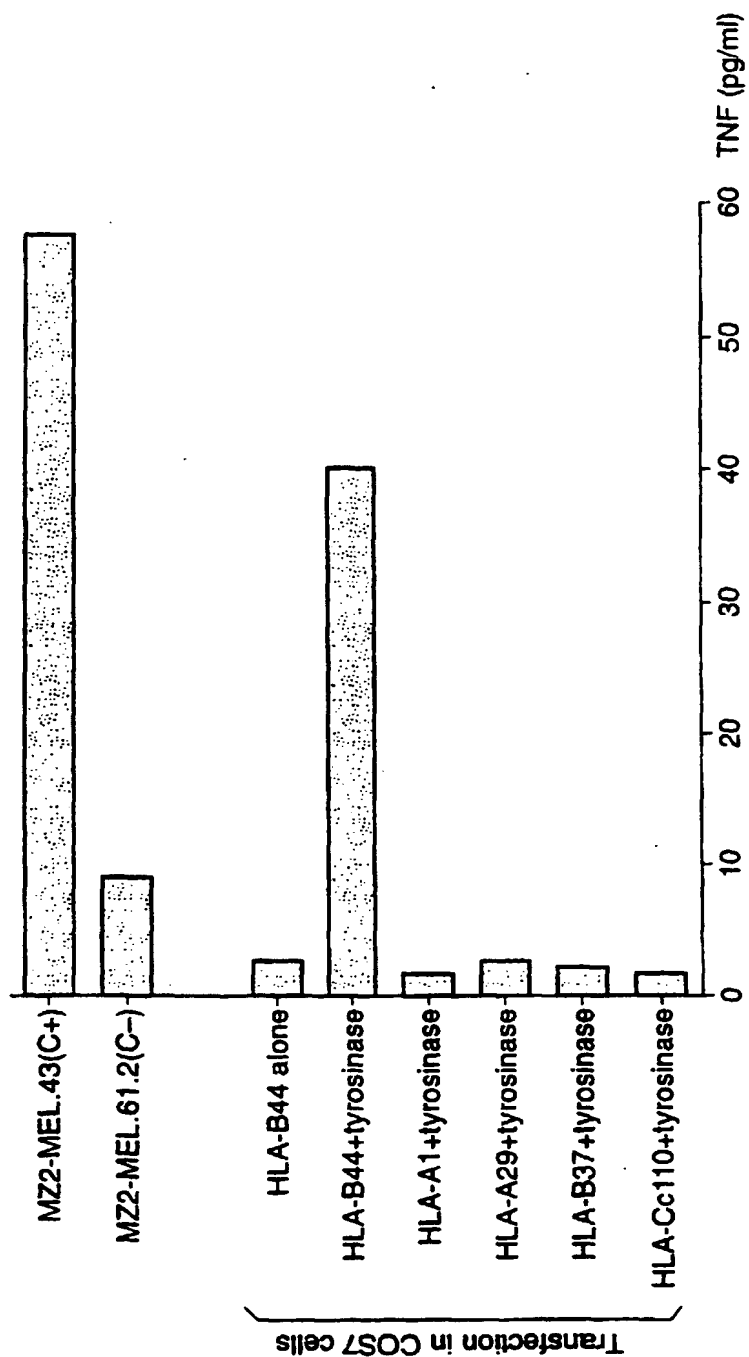


FIG. 6





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 20 4249

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	WO 90 12869 A (SLOAN KETTERING INST CANCER) 1 November 1990 (1990-11-01)		A61K35/12 C12Q1/00 C12Q1/02 C12N5/10 C12N15/00 C12N15/52 C07K7/06
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
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The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 24 May 2000	Examiner Deffner, C-A
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03.92 (P4/C01)

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

EP 99 20 4249

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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24-05-2000

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FIG. 1A

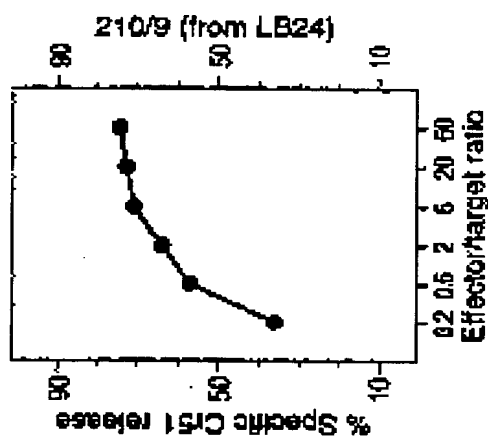


FIG. 1B

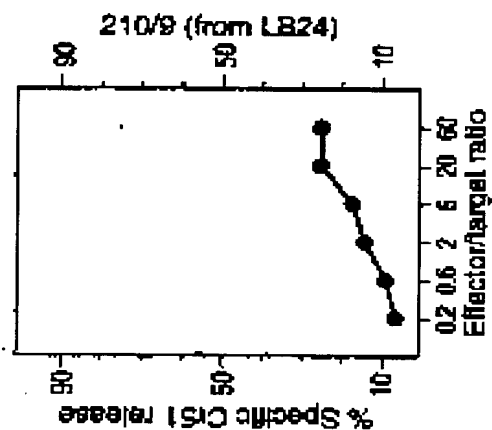


FIG. 1C

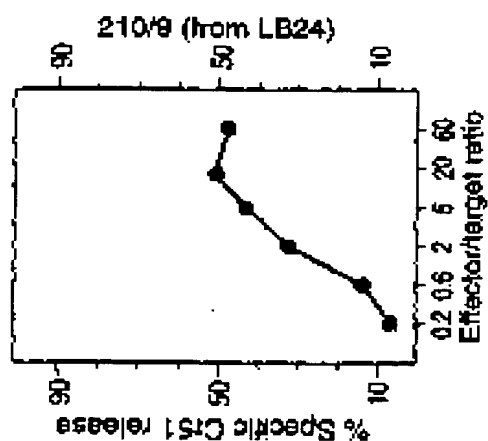


FIG. 1A'

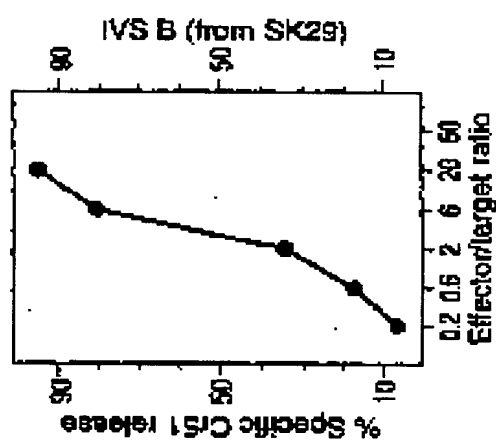


FIG. 1B'

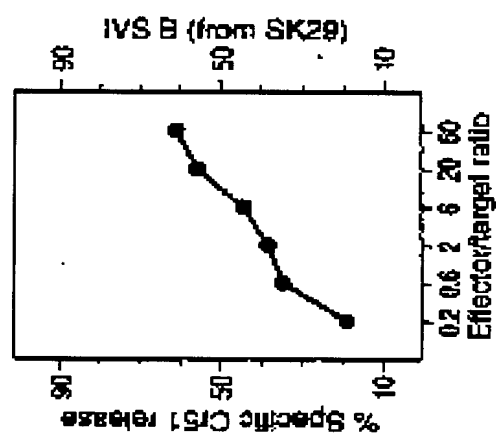
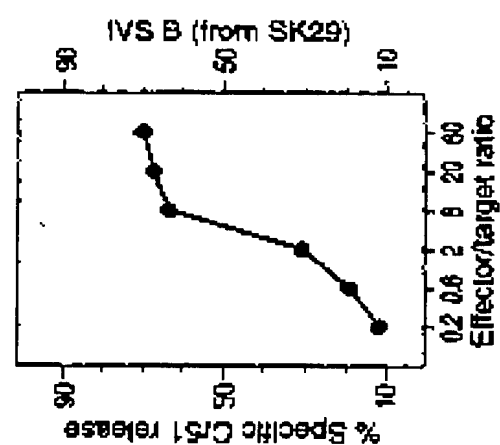


FIG. 1C'



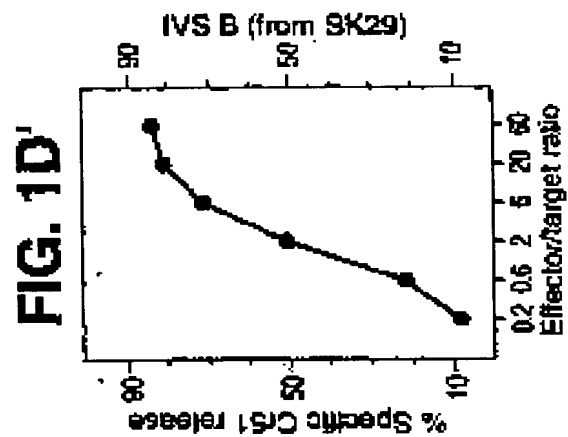
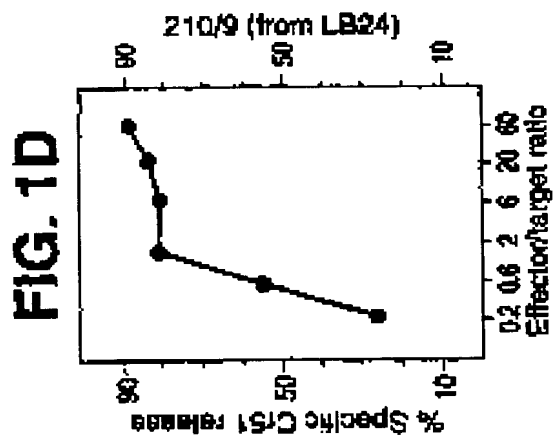
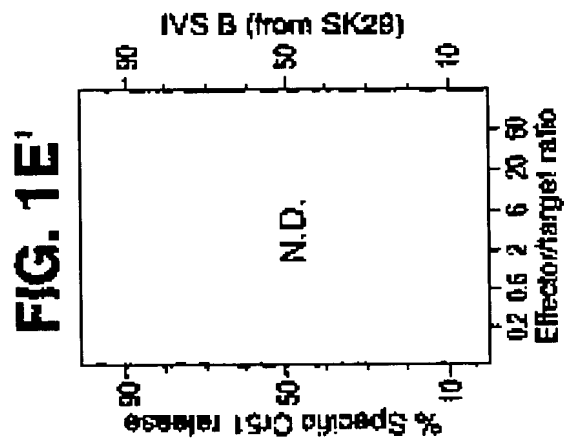
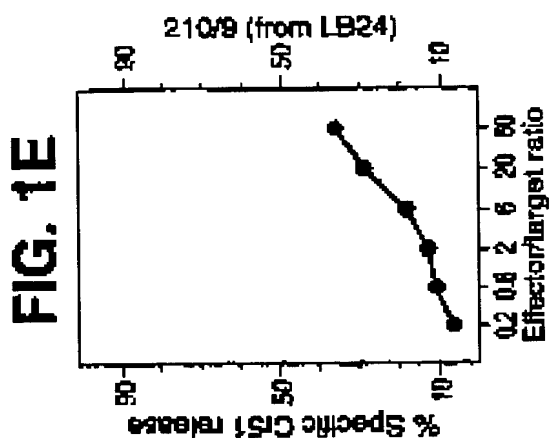


Fig. 1

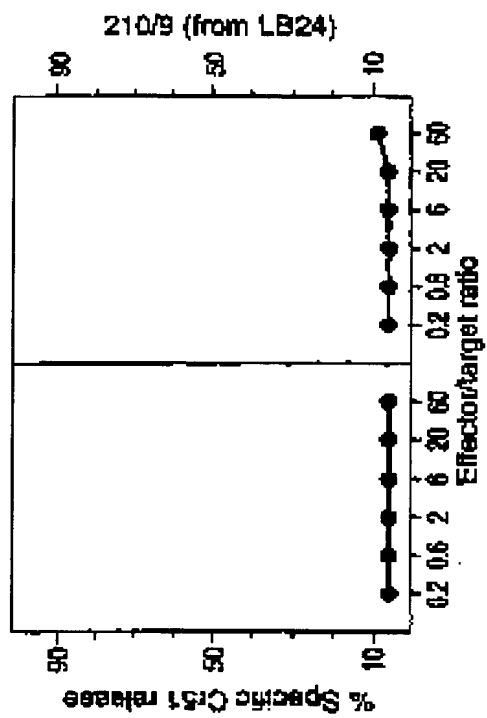
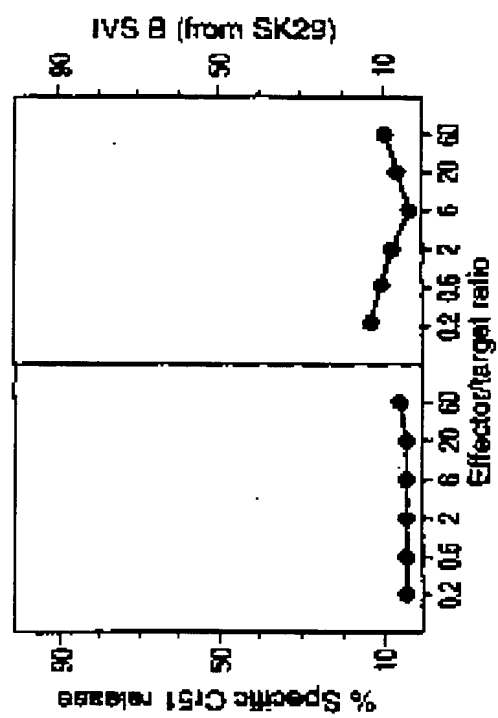
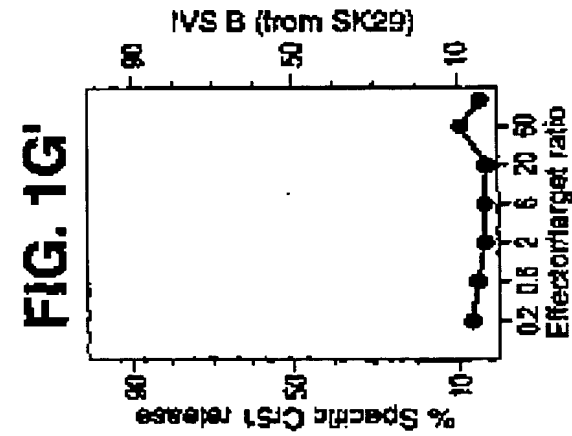
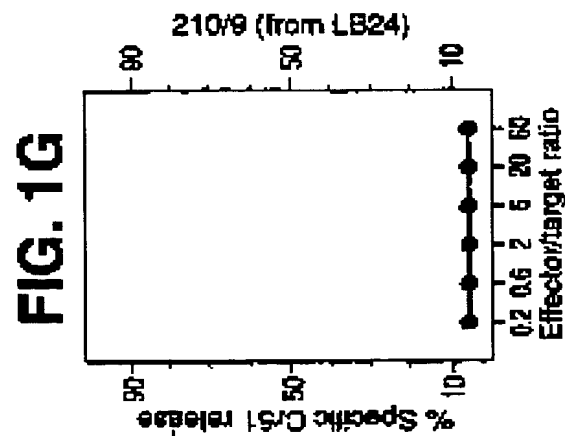
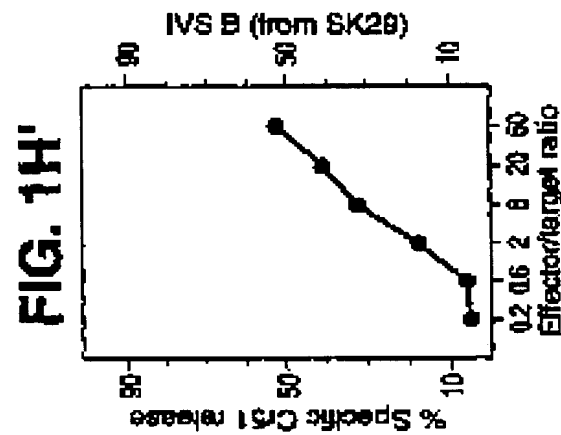
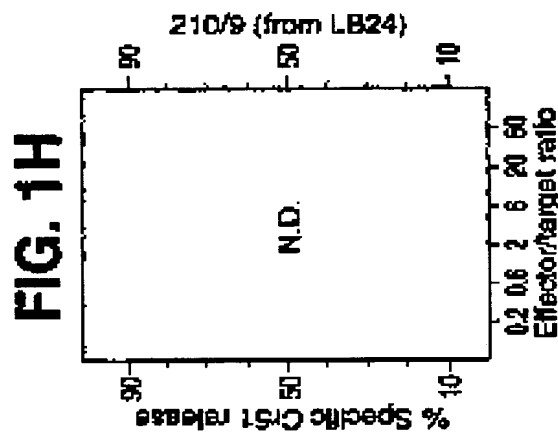
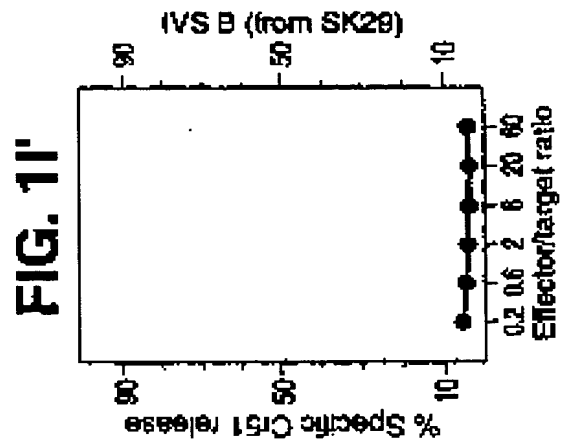
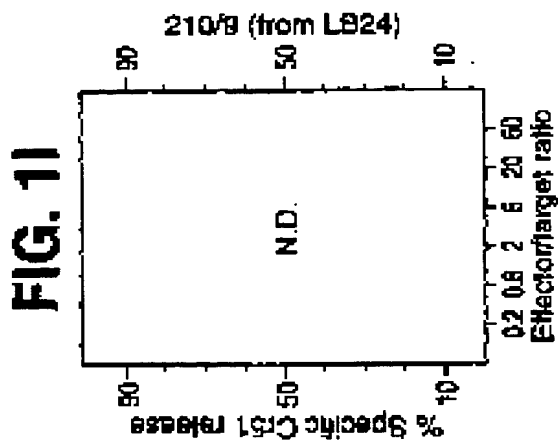


FIG. 1F





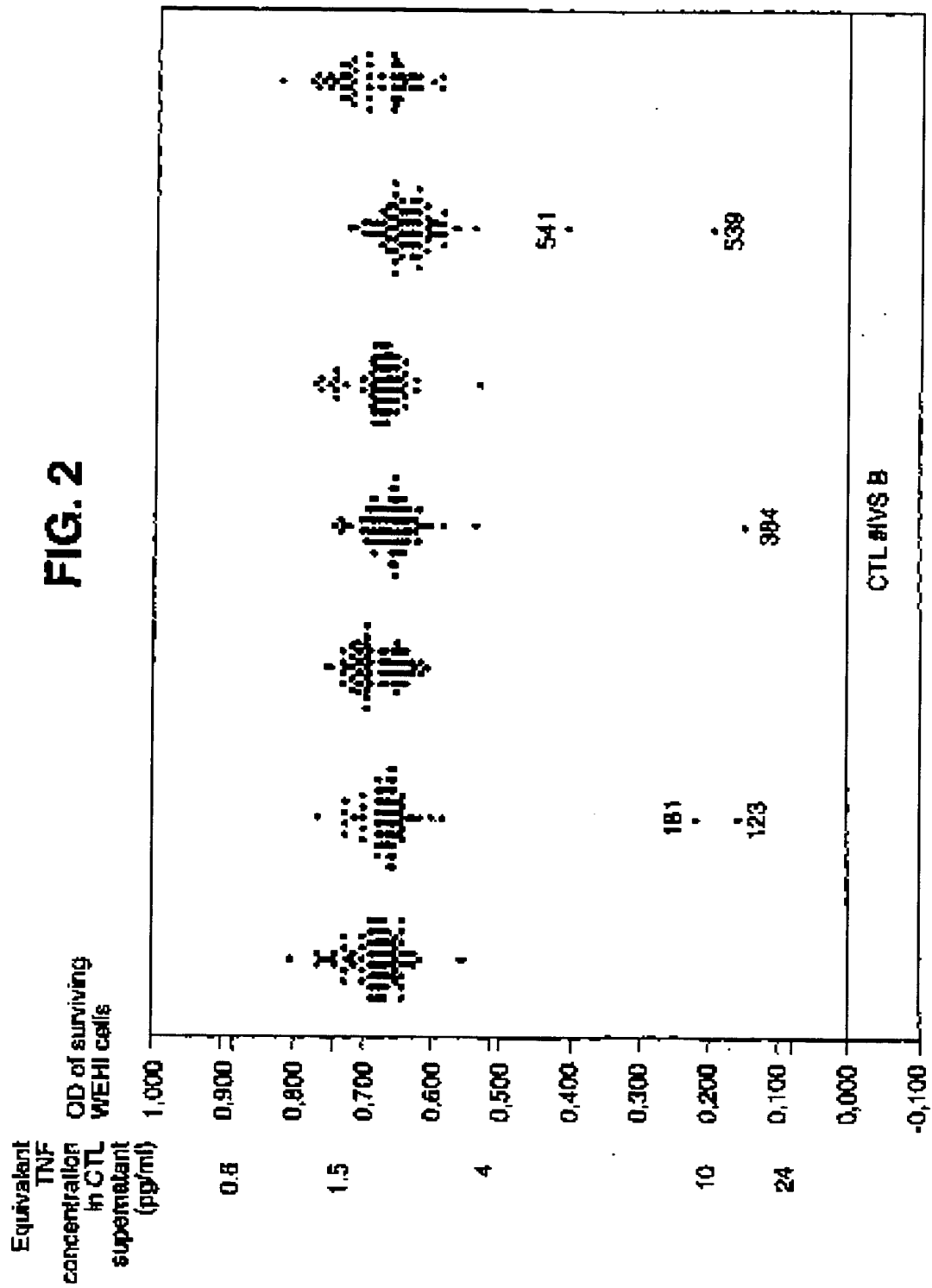


FIG. 3

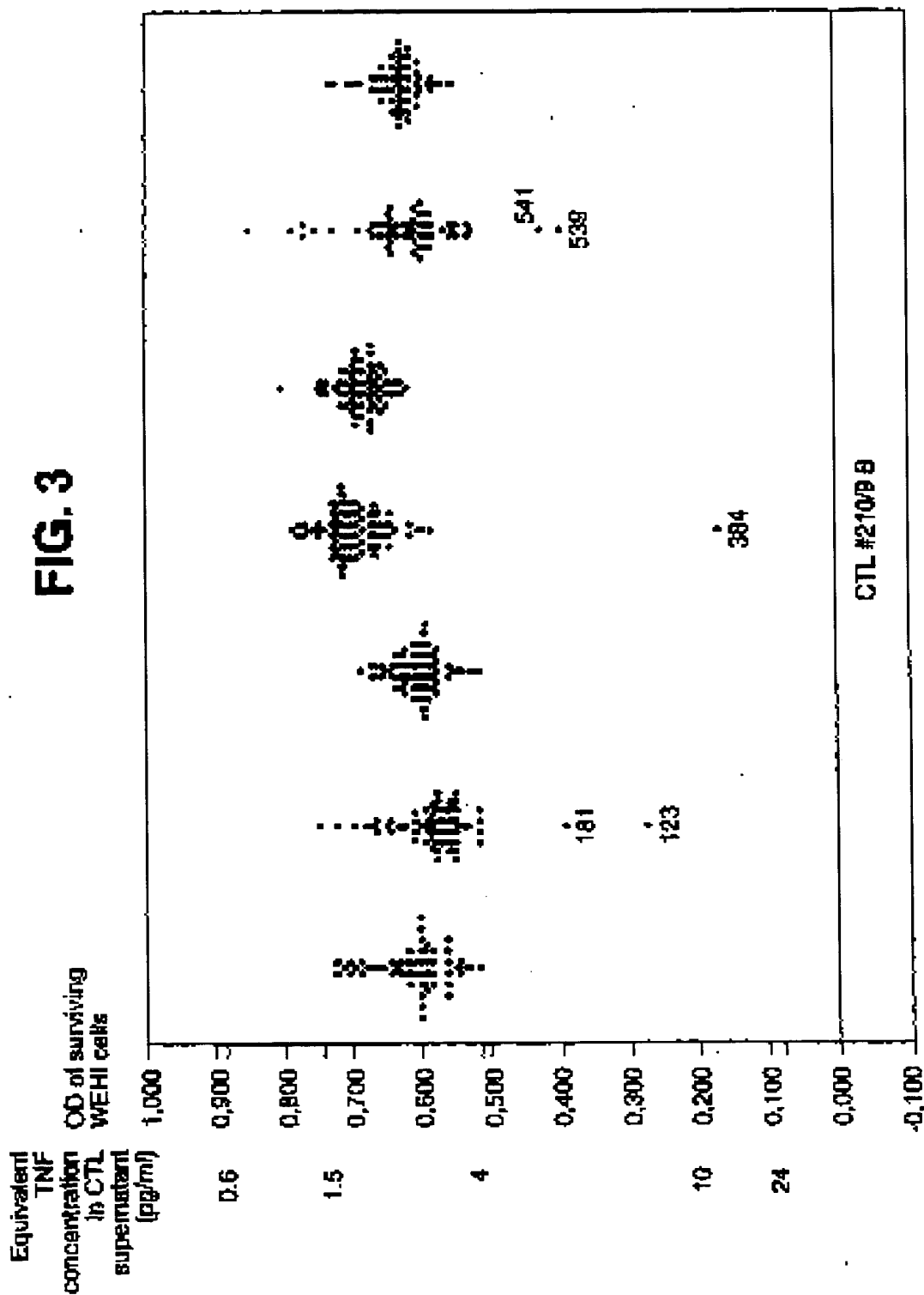


FIG. 4A

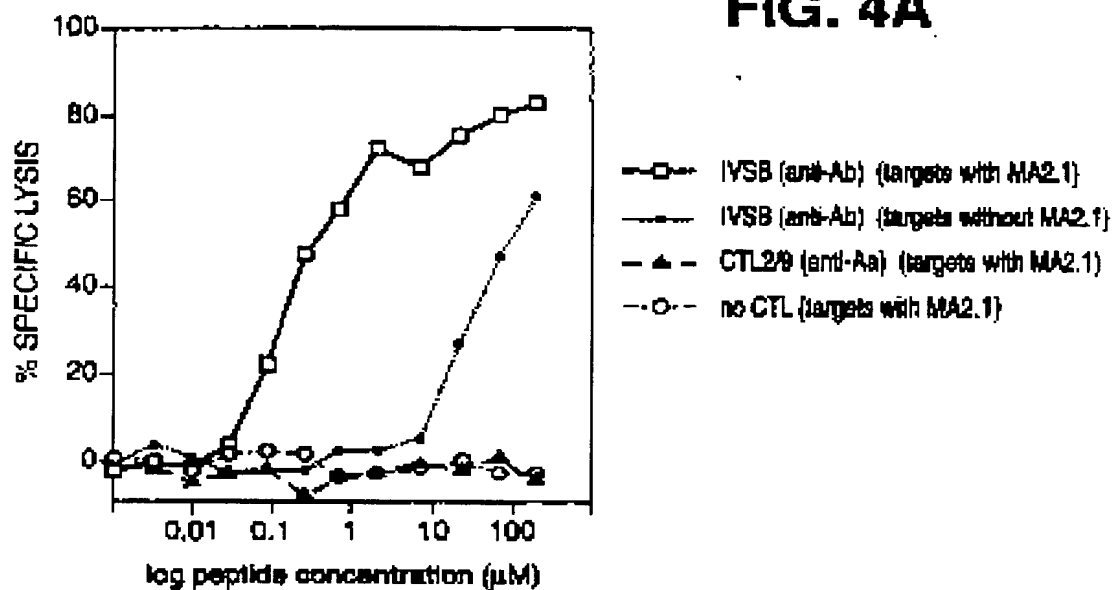


FIG. 4B

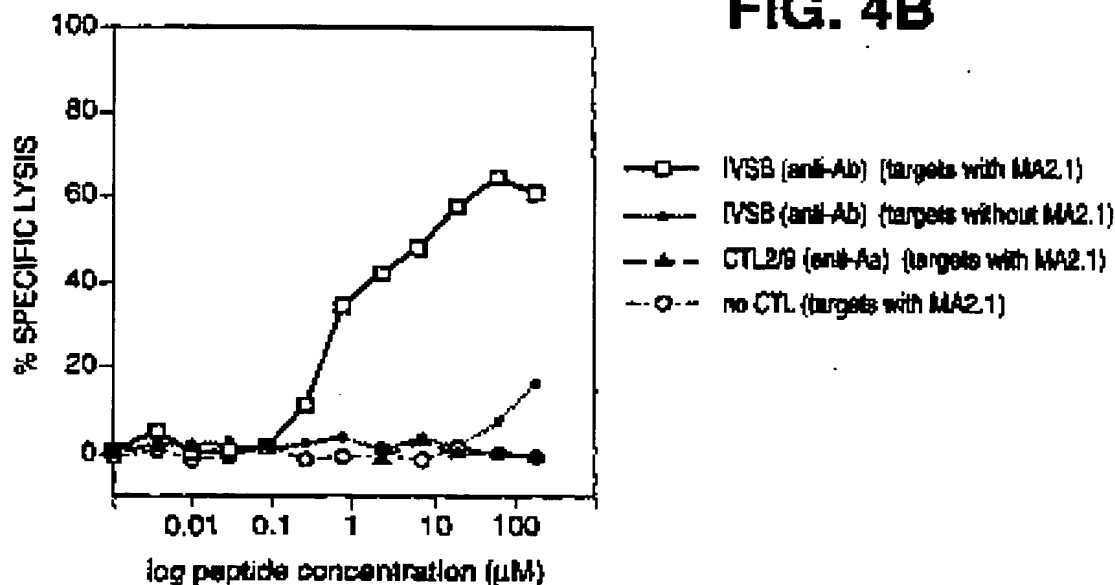


FIG. 5

